

## NUCLEIC ACID SEQUENCES TO PROTEINS INVOLVED IN TOCOPHEROL SYNTHESIS

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### INTRODUCTION

This application claims the benefit of the filing date of the provisional Application U.S. Serial Number 60/129,899, filed April 15, 1999, and the provisional Application, U.S. Serial  
10 Number 60/146,461, filed July 30, 1999.

### TECHNICAL FIELD

The present invention is directed to nucleic acid and amino acid sequences and constructs, and methods related thereto.

### BACKGROUND

Isoprenoids are ubiquitous compounds found in all living organisms. Plants synthesize a diverse array of greater than 22,000 isoprenoids (Connolly and Hill (1992) *Dictionary of Terpenoids*, Chapman and Hall, New York, NY). In plants, isoprenoids play essential roles in particular cell functions such as production of sterols, contributing to eukaryotic membrane architecture, acyclic polyprenoids found in the side chain of ubiquinone and plastoquinone, growth regulators like abscisic acid, gibberellins, brassinosteroids or the photosynthetic pigments chlorophylls and carotenoids. Although the physiological role of other plant isoprenoids is less evident, like that of the vast array of secondary metabolites, some are known to play key roles  
25 mediating the adaptative responses to different environmental challenges. In spite of the remarkable diversity of structure and function, all isoprenoids originate from a single metabolic precursor, isopentenyl diphosphate (IPP) (Wright, (1961) *Annu. Rev. Biochem.* 20:525-548; and Spurgeon and Porter, (1981) in Biosynthesis of Isoprenoid Compounds, Porter and Spurgeon eds (John Wiley, New York) Vol. 1, pp1-46).

A number of unique and interconnected biochemical pathways derived from the isoprenoid pathway leading to secondary metabolites, including tocopherols, exist in chloroplasts of higher plants. Tocopherols not only perform vital functions in plants, but are also important from mammalian nutritional perspectives. In plastids, tocopherols account for up to 40% of the total quinone pool.

Tocopherols and tocotrienols (unsaturated tocopherol derivatives) are well known antioxidants, and play an important role in protecting cells from free radical damage, and in the prevention of many diseases, including cardiac disease, cancer, cataracts, retinopathy, Alzheimer's disease, and neurodegeneration, and have been shown to have beneficial effects on symptoms of arthritis, and in anti-aging. Vitamin E is used in chicken feed for improving the shelf life, appearance, flavor, and oxidative stability of meat, and to transfer tocopherols from feed to eggs. Vitamin E has been shown to be essential for normal reproduction, improves overall performance, and enhances immunocompetence in livestock animals. Vitamin E supplement in animal feed also imparts oxidative stability to milk products.

The demand for natural tocopherols as supplements has been steadily growing at a rate of 10-20% for the past three years. At present, the demand exceeds the supply for natural tocopherols, which are known to be more biopotent than racemic mixtures of synthetically produced tocopherols. Naturally occurring tocopherols are all *d*-stereoisomers, whereas synthetic  $\alpha$ -tocopherol is a mixture of eight *d,l*- $\alpha$ -tocopherol isomers, only one of which (12.5%) is identical to the natural *d*- $\alpha$ -tocopherol. Natural *d*- $\alpha$ -tocopherol has the highest vitamin E activity (1.49 IU/mg) when compared to other natural tocopherols or tocotrienols. The synthetic  $\alpha$ -tocopherol has a vitamin E activity of 1.1 IU/mg. In 1995, the worldwide market for raw refined tocopherols was \$1020 million; synthetic materials comprised 85-88% of the market, the remaining 12-15% being natural materials. The best sources of natural tocopherols and tocotrienols are vegetable oils and grain products. Currently, most of the natural Vitamin E is produced from  $\gamma$ -tocopherol derived from soy oil processing, which is subsequently converted to  $\alpha$ -tocopherol by chemical modification ( $\alpha$ -tocopherol exhibits the greatest biological activity).

Methods of enhancing the levels of tocopherols and tocotrienols in plants, especially levels of the more desirable compounds that can be used directly, without chemical modification, would be useful to the art as such molecules exhibit better functionality and bioavailability.

In addition, methods for the increased production of other isoprenoid derived compounds in a host plant cell is desirable. Furthermore, methods for the production of particular isoprenoid compounds in a host plant cell is also needed.

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## SUMMARY OF THE INVENTION

The present invention is directed to prenyltransferase (PT), and in particular to PT polynucleotides and polypeptides. The polynucleotides and polypeptides of the present invention include those derived from prokaryotic and eukaryotic sources.

Thus, one aspect of the present invention relates to isolated polynucleotide sequences encoding prenyltransferase proteins. In particular, isolated nucleic acid sequences encoding PT proteins from bacterial and plant sources are provided.

Another aspect of the present invention relates to oligonucleotides which include partial or complete PT encoding sequences.

It is also an aspect of the present invention to provide recombinant DNA constructs which can be used for transcription or transcription and translation (expression) of prenyltransferase. In particular, constructs are provided which are capable of transcription or transcription and translation in host cells.

In another aspect of the present invention, methods are provided for production of prenyltransferase in a host cell or progeny thereof. In particular, host cells are transformed or transfected with a DNA construct which can be used for transcription or transcription and translation of prenyltransferase. The recombinant cells which contain prenyltransferase are also part of the present invention.

In a further aspect, the present invention relates to methods of using polynucleotide and polypeptide sequences to modify the tocopherol content of host cells, particularly in host plant cells. Plant cells having such a modified tocopherol content are also contemplated herein.

The modified plants, seeds and oils obtained by the expression of the prenyltransferases are also considered part of the invention.

## BRIEF DESCRIPTION OF THE DRAWINGS

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Figure 1 provides an amino acid sequence alignment between ATPT2, ATPT3, ATPT4, ATPT8, and ATPT12 are performed using ClustalW.

5 Figure 2 provides a schematic picture of the expression construct pCGN10800.

Figure 3 provides a schematic picture of the expression construct pCGN10801.

Figure 4 provides a schematic picture of the expression construct pCGN10803.

Figure 5 provides a schematic picture of the expression construct pCGN10806.

Figure 6 provides a schematic picture of the expression construct pCGN10807.

10 Figure 7 provides a schematic picture of the expression construct pCGN10808.

Figure 8 provides a schematic picture of the expression construct pCGN10809.

Figure 9 provides a schematic picture of the expression construct pCGN10810.

Figure 10 provides a schematic picture of the expression construct pCGN10811.

Figure 11 provides a schematic picture of the expression construct pCGN10812.

Figure 12 provides a schematic picture of the expression construct pCGN10813.

Figure 13 provides a schematic picture of the expression construct pCGN10814.

Figure 14 provides a schematic picture of the expression construct pCGN10815.

Figure 15 provides a schematic picture of the expression construct pCGN10816.

Figure 16 provides a schematic picture of the expression construct pCGN10817.

Figure 17 provides a schematic picture of the expression construct pCGN10819.

Figure 18 provides a schematic picture of the expression construct pCGN10824.

Figure 19 provides a schematic picture of the expression construct pCGN10825.

Figure 20 provides a schematic picture of the expression construct pCGN10826.

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25 ~~Figure 21 provides an amino acid sequence alignment using ClustalW between the *Synechocystis* sequence knockouts.~~

Figure 22 provides an amino acid sequence of the ATPT2, ATPT3, ATPT4, ATPT8, and ATPT12 protein sequences from *Arabidopsis* and the slr1736, slr0926, slr1899, slr0056, and the slr1518 amino acid sequences from *Synechocystis*.

30 Figure 23 provides the results of the enzymatic assay from preparations of wild type *Synechocystis* strain 6803, and *Synechocystis* slr1736 knockout.

Figure 24 provides bar graphs of HPLC data obtained from seed extracts of transgenic *Arabidopsis* containing pCGN10822, which provides of the expression of the ATPT2 sequence, in the sense orientation, from the napin promoter. Provided are graphs for alpha, gamma, and delta tocopherols, as well as total tocopherol for 22 transformed lines, as well as a nontransformed (wildtype) control.

Figure 25 provides a bar graph of HPLC analysis of seed extracts from *Arabidopsis* plants transformed with pCGN10803 (35S-ATPT2, in the antisense orientation), pCGN10802 (line 1625, napin ATPT2 in the sense orientation), pCGN10809 (line 1627, 35S-ATPT3 in the sense orientation), a nontransformed (wt) control, and a empty vector transformed control.

## DETAILED DESCRIPTION OF THE INVENTION

The present invention provides, *inter alia*, compositions and methods for altering (for example, increasing and decreasing) the tocopherol levels and/or modulating their ratios in host cells. In particular, the present invention provides polynucleotides, polypeptides, and methods of use thereof for the modulation of tocopherol content in host plant cells.

The present invention provides polynucleotide and polypeptide sequences involved in the prenylation of straight chain and aromatic compounds. Straight chain prenyl transferases as used herein comprises sequences which encode proteins involved in the prenylation of straight chain compounds, including, but not limited to, geranyl geranyl pyrophosphate and farnesyl pyrophosphate. Aromatic prenyl transferases, as used herein, comprises sequences which encode proteins involved in the prenylation of aromatic compounds, including, but not limited to, menaquinone, ubiquinone, chlorophyll, and homogentisic acid. The prenyl transferase of the present invention preferably prenylates homogentisic acid.

The biosynthesis of  $\alpha$ -tocopherol in higher plants involves condensation of homogentisic acid and phytylpyrophosphate to form 2-methyl-6-phytylbenzoquinol that can, by cyclization and subsequent methylations (Fiedler et al., 1982, *Planta*, 155: 511-515, Soll et al., 1980, *Arch. Biochem. Biophys.* 204: 544-550, Marshall et al., 1985 *Phytochem.*, 24: 1705-1711, all of which are herein incorporated by reference in their entirety), form various tocopherols. The *Arabidopsis*

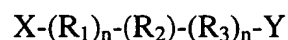
*pds2* mutant identified and characterized by Norris *et al.* (1995), is deficient in tocopherol and plastiquinone-9 accumulation. Further genetic and biochemical analysis suggests that the protein encoded by *PDS2* may be responsible for the prenylation of homogentisic acid. This may be a rate limiting step in tocopherol biosynthesis, and this gene has yet to be isolated. Thus, it is an aspect of the present invention to provide polynucleotides and polypeptides involved in the prenylation of homogentisic acid.

### Isolated Polynucleotides, Proteins, and Polypeptides

A first aspect of the present invention relates to isolated prenyltransferase polynucleotides. The polynucleotide sequences of the present invention include isolated polynucleotides that encode the polypeptides of the invention having a deduced amino acid sequence selected from the group of sequences set forth in the Sequence Listing and to other polynucleotide sequences closely related to such sequences and variants thereof.

The invention provides a polynucleotide sequence identical over its entire length to each coding sequence as set forth in the Sequence Listing. The invention also provides the coding sequence for the mature polypeptide or a fragment thereof, as well as the coding sequence for the mature polypeptide or a fragment thereof in a reading frame with other coding sequences, such as those encoding a leader or secretory sequence, a pre-, pro-, or prepro- protein sequence. The polynucleotide can also include non-coding sequences, including for example, but not limited to, non-coding 5' and 3' sequences, such as the transcribed, untranslated sequences, termination signals, ribosome binding sites, sequences that stabilize mRNA, introns, polyadenylation signals, and additional coding sequence that encodes additional amino acids. For example, a marker sequence can be included to facilitate the purification of the fused polypeptide. Polynucleotides of the present invention also include polynucleotides comprising a structural gene and the naturally associated sequences that control gene expression.

The invention also includes polynucleotides of the formula:



wherein, at the 5' end, X is hydrogen, and at the 3' end, Y is hydrogen or a metal,  $R_1$  and  $R_3$  are any nucleic acid residue, n is an integer between 1 and 3000, preferably between 1 and 1000 and

R<sub>2</sub> is a nucleic acid sequence of the invention, particularly a nucleic acid sequence selected from the group set forth in the Sequence Listing and preferably those of SEQ IDNOs: 1, 3, 5, 7, 8, 10, 11, 13-16, 18, 23, 29, 36, and 38. In the formula, R<sub>2</sub> is oriented so that its 5' end residue is at the left, bound to R<sub>1</sub>, and its 3' end residue is at the right, bound to R<sub>3</sub>. Any stretch of nucleic acid residues denoted by either R group, where R is greater than 1, may be either a heteropolymer or a homopolymer, preferably a heteropolymer.

The invention also relates to variants of the polynucleotides described herein that encode for variants of the polypeptides of the invention. Variants that are fragments of the polynucleotides of the invention can be used to synthesize full-length polynucleotides of the invention. Preferred embodiments are polynucleotides encoding polypeptide variants wherein 5 to 10, 1 to 5, 1 to 3, 2, 1 or no amino acid residues of a polypeptide sequence of the invention are substituted, added or deleted, in any combination. Particularly preferred are substitutions, additions, and deletions that are silent such that they do not alter the properties or activities of the polynucleotide or polypeptide.

Further preferred embodiments of the invention that are at least 50%, 60%, or 70% identical over their entire length to a polynucleotide encoding a polypeptide of the invention, and polynucleotides that are complementary to such polynucleotides. More preferable are polynucleotides that comprise a region that is at least 80% identical over its entire length to a polynucleotide encoding a polypeptide of the invention and polynucleotides that are complementary thereto. In this regard, polynucleotides at least 90% identical over their entire length are particularly preferred, those at least 95% identical are especially preferred. Further, those with at least 97% identity are highly preferred and those with at least 98% and 99% identity are particularly highly preferred, with those at least 99% being the most highly preferred.

Preferred embodiments are polynucleotides that encode polypeptides that retain substantially the same biological function or activity as the mature polypeptides encoded by the polynucleotides set forth in the Sequence Listing.

The invention further relates to polynucleotides that hybridize to the above-described sequences. In particular, the invention relates to polynucleotides that hybridize under stringent conditions to the above-described polynucleotides. As used herein, the terms "stringent conditions" and "stringent hybridization conditions" mean that hybridization will generally occur

if there is at least 95% and preferably at least 97% identity between the sequences. An example of stringent hybridization conditions is overnight incubation at 42°C in a solution comprising 50% formamide, 5x SSC (150 mM NaCl, 15 mM trisodium citrate), 50 mM sodium phosphate (pH 7.6), 5x Denhardt's solution, 10% dextran sulfate, and 20 micrograms/milliliter denatured, sheared salmon sperm DNA, followed by washing the hybridization support in 0.1x SSC at approximately 65°C. Other hybridization and wash conditions are well known and are exemplified in Sambrook, *et al.*, Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor, NY (1989), particularly Chapter 11.

The invention also provides a polynucleotide consisting essentially of a polynucleotide sequence obtainable by screening an appropriate library containing the complete gene for a polynucleotide sequence set forth in the Sequence Listing under stringent hybridization conditions with a probe having the sequence of said polynucleotide sequence or a fragment thereof; and isolating said polynucleotide sequence. Fragments useful for obtaining such a polynucleotide include, for example, probes and primers as described herein.

As discussed herein regarding polynucleotide assays of the invention, for example, polynucleotides of the invention can be used as a hybridization probe for RNA, cDNA, or genomic DNA to isolate full length cDNAs or genomic clones encoding a polypeptide and to isolate cDNA or genomic clones of other genes that have a high sequence similarity to a polynucleotide set forth in the Sequence Listing. Such probes will generally comprise at least 15 bases. Preferably such probes will have at least 30 bases and can have at least 50 bases. Particularly preferred probes will have between 30 bases and 50 bases, inclusive.

The coding region of each gene that comprises or is comprised by a polynucleotide sequence set forth in the Sequence Listing may be isolated by screening using a DNA sequence provided in the Sequence Listing to synthesize an oligonucleotide probe. A labeled oligonucleotide having a sequence complementary to that of a gene of the invention is then used to screen a library of cDNA, genomic DNA or mRNA to identify members of the library which hybridize to the probe. For example, synthetic oligonucleotides are prepared which correspond to the prenyltransferase EST sequences. The oligonucleotides are used as primers in polymerase chain reaction (PCR) techniques to obtain 5' and 3' terminal sequence of prenyl transferase genes. Alternatively, where oligonucleotides of low degeneracy can be prepared from particular



prenyltransferase peptides, such probes may be used directly to screen gene libraries for prenyltransferase gene sequences. In particular, screening of cDNA libraries in phage vectors is useful in such methods due to lower levels of background hybridization.

Typically, a prenyltransferase sequence obtainable from the use of nucleic acid probes will show 60-70% sequence identity between the target prenyltransferase sequence and the encoding sequence used as a probe. However, lengthy sequences with as little as 50-60% sequence identity may also be obtained. The nucleic acid probes may be a lengthy fragment of the nucleic acid sequence, or may also be a shorter, oligonucleotide probe. When longer nucleic acid fragments are employed as probes (greater than about 100 bp), one may screen at lower stringencies in order to obtain sequences from the target sample which have 20-50% deviation (i.e., 50-80% sequence homology) from the sequences used as probe. Oligonucleotide probes can be considerably shorter than the entire nucleic acid sequence encoding an prenyltransferase enzyme, but should be at least about 10, preferably at least about 15, and more preferably at least about 20 nucleotides. A higher degree of sequence identity is desired when shorter regions are used as opposed to longer regions. It may thus be desirable to identify regions of highly conserved amino acid sequence to design oligonucleotide probes for detecting and recovering other related prenyltransferase genes. Shorter probes are often particularly useful for polymerase chain reactions (PCR), especially when highly conserved sequences can be identified. (See, Gould, *et al.*, *PNAS USA* (1989) 86:1934-1938.).

Another aspect of the present invention relates to prenyltransferase polypeptides. Such polypeptides include isolated polypeptides set forth in the Sequence Listing, as well as polypeptides and fragments thereof, particularly those polypeptides which exhibit prenyltransferase activity and also those polypeptides which have at least 50%, 60% or 70% identity, preferably at least 80% identity, more preferably at least 90% identity, and most preferably at least 95% identity to a polypeptide sequence selected from the group of sequences set forth in the Sequence Listing, and also include portions of such polypeptides, wherein such portion of the polypeptide preferably includes at least 30 amino acids and more preferably includes at least 50 amino acids.

"Identity", as is well understood in the art, is a relationship between two or more polypeptide sequences or two or more polynucleotide sequences, as determined by comparing the

sequences. In the art, "identity" also means the degree of sequence relatedness between polypeptide or polynucleotide sequences, as determined by the match between strings of such sequences. "Identity" can be readily calculated by known methods including, but not limited to, those described in *Computational Molecular Biology*, Lesk, A.M., ed., Oxford University Press, New York (1988); *Biocomputing: Informatics and Genome Projects*, Smith, D.W., ed., Academic Press, New York, 1993; *Computer Analysis of Sequence Data, Part I*, Griffin, A.M. and Griffin, H.G., eds., Humana Press, New Jersey (1994); *Sequence Analysis in Molecular Biology*, von Heinje, G., Academic Press (1987); *Sequence Analysis Primer*, Gribskov, M. and Devereux, J., eds., Stockton Press, New York (1991); and Carillo, H., and Lipman, D., *SIAM J Applied Math*, 48:1073 (1988). Methods to determine identity are designed to give the largest match between the sequences tested. Moreover, methods to determine identity are codified in publicly available programs. Computer programs which can be used to determine identity between two sequences include, but are not limited to, GCG (Devereux, J., et al., *Nucleic Acids Research* 12(1):387 (1984); suite of five BLAST programs, three designed for nucleotide sequences queries (BLASTN, BLASTX, and TBLASTX) and two designed for protein sequence queries (BLASTP and TBLASTN) (Coulson, *Trends in Biotechnology*, 12: 76-80 (1994); Birren, et al., *Genome Analysis*, 1: 543-559 (1997)). The BLAST X program is publicly available from NCBI and other sources (*BLAST Manual*, Altschul, S., et al., NCBI NLM NIH, Bethesda, MD 20894; Altschul, S., et al., *J. Mol. Biol.*, 215:403-410 (1990)). The well known Smith Waterman algorithm can also be used to determine identity.

Parameters for polypeptide sequence comparison typically include the following:

Algorithm: Needleman and Wunsch, *J. Mol. Biol.* 48:443-453 (1970)

Comparison matrix: BLOSSUM62 from Hentikoff and Hentikoff, *Proc. Natl. Acad. Sci USA* 89:10915-10919 (1992)

Gap Penalty: 12

Gap Length Penalty: 4

A program which can be used with these parameters is publicly available as the "gap" program from Genetics Computer Group, Madison Wisconsin. The above parameters along with no penalty for end gap are the default parameters for peptide comparisons.

Parameters for polynucleotide sequence comparison include the following:

Algorithm: Needleman and Wunsch, J. Mol. Biol. 48:443-453 (1970)

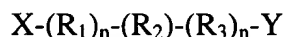
Comparison matrix: matches = +10; mismatches = 0

Gap Penalty: 50

Gap Length Penalty: 3

5 A program which can be used with these parameters is publicly available as the "gap" program from Genetics Computer Group, Madison Wisconsin. The above parameters are the default parameters for nucleic acid comparisons.

The invention also includes polypeptides of the formula:



10 wherein, at the amino terminus, X is hydrogen, and at the carboxyl terminus, Y is hydrogen or a metal,  $R_1$  and  $R_3$  are any amino acid residue,  $n$  is an integer between 1 and 1000, and  $R_2$  is an amino acid sequence of the invention, particularly an amino acid sequence selected from the group set forth in the Sequence Listing and preferably those encoded by the sequences provided in SEQ ID NOs: 2, 4, 6, 9, 12, 17, 19-22, 24-28, 30, 32-35, 37, and 39. In the formula,  $R_2$  is oriented so that its amino terminal residue is at the left, bound to  $R_1$ , and its carboxy terminal residue is at the right, bound to  $R_3$ . Any stretch of amino acid residues denoted by either R group, where R is greater than 1, may be either a heteropolymer or a homopolymer, preferably a heteropolymer.

Polypeptides of the present invention include isolated polypeptides encoded by a polynucleotide comprising a sequence selected from the group of a sequence contained in the Sequence Listing set forth herein.

The polypeptides of the present invention can be mature protein or can be part of a fusion protein.

25 Fragments and variants of the polypeptides are also considered to be a part of the invention. A fragment is a variant polypeptide which has an amino acid sequence that is entirely the same as part but not all of the amino acid sequence of the previously described polypeptides. The fragments can be "free-standing" or comprised within a larger polypeptide of which the fragment forms a part or a region, most preferably as a single continuous region. Preferred fragments are biologically active fragments which are those fragments that mediate activities of  
30 the polypeptides of the invention, including those with similar activity or improved activity or

with a decreased activity. Also included are those fragments that antigenic or immunogenic in an animal, particularly a human.

Variants of the polypeptide also include polypeptides that vary from the sequences set forth in the Sequence Listing by conservative amino acid substitutions, substitution of a residue  
5 by another with like characteristics. In general, such substitutions are among Ala, Val, Leu and Ile; between Ser and Thr; between Asp and Glu; between Asn and Gln; between Lys and Arg; or between Phe and Tyr. Particularly preferred are variants in which 5 to 10; 1 to 5; 1 to 3 or one amino acid(s) are substituted, deleted, or added, in any combination.

Variants that are fragments of the polypeptides of the invention can be used to produce  
10 the corresponding full length polypeptide by peptide synthesis. Therefore, these variants can be used as intermediates for producing the full-length polypeptides of the invention.

The polynucleotides and polypeptides of the invention can be used, for example, in the transformation of host cells, such as plant host cells, as further discussed herein.

The invention also provides polynucleotides that encode a polypeptide that is a mature protein plus additional amino or carboxyl-terminal amino acids, or amino acids within the mature polypeptide (for example, when the mature form of the protein has more than one polypeptide chain). Such sequences can, for example, play a role in the processing of a protein from a precursor to a mature form, allow protein transport, shorten or lengthen protein half-life, or facilitate manipulation of the protein in assays or production. It is contemplated that cellular  
20 enzymes can be used to remove any additional amino acids from the mature protein.

A precursor protein, having the mature form of the polypeptide fused to one or more prosequences may be an inactive form of the polypeptide. The inactive precursors generally are activated when the prosequences are removed. Some or all of the prosequences may be removed prior to activation. Such precursor protein are generally called proproteins.

## **Plant Constructs and Methods of Use**

Of particular interest is the use of the nucleotide sequences in recombinant DNA  
30 constructs to direct the transcription or transcription and translation (expression) of the prenyltransferase sequences of the present invention in a host plant cell. The expression

constructs generally comprise a promoter functional in a host plant cell operably linked to a nucleic acid sequence encoding a prenyltransferase of the present invention and a transcriptional termination region functional in a host plant cell.

A first nucleic acid sequence is "operably linked" or "operably associated" with a second nucleic acid sequence when the sequences are so arranged that the first nucleic acid sequence affects the function of the second nucleic-acid sequence. Preferably, the two sequences are part of a single contiguous nucleic acid molecule and more preferably are adjacent. For example, a promoter is operably linked to a gene if the promoter regulates or mediates transcription of the gene in a cell.

Those skilled in the art will recognize that there are a number of promoters which are functional in plant cells, and have been described in the literature. Chloroplast and plastid specific promoters, chloroplast or plastid functional promoters, and chloroplast or plastid operable promoters are also envisioned.

One set of plant functional promoters are constitutive promoters such as the CaMV35S or FMV35S promoters that yield high levels of expression in most plant organs. Enhanced or duplicated versions of the CaMV35S and FMV35S promoters are useful in the practice of this invention (Odell, *et al.* (1985) *Nature* 313:810-812; Rogers, U.S. Patent Number 5,378, 619). In addition, it may also be preferred to bring about expression of the prenyltransferase gene in specific tissues of the plant, such as leaf, stem, root, tuber, seed, fruit, etc., and the promoter chosen should have the desired tissue and developmental specificity.

Of particular interest is the expression of the nucleic acid sequences of the present invention from transcription initiation regions which are preferentially expressed in a plant seed tissue. Examples of such seed preferential transcription initiation sequences include those sequences derived from sequences encoding plant storage protein genes or from genes involved in fatty acid biosynthesis in oilseeds. Examples of such promoters include the 5' regulatory regions from such genes as napin (Kridl *et al.*, *Seed Sci. Res.* 1:209:219 (1991)), phaseolin, zein, soybean trypsin inhibitor, ACP, stearyl-ACP desaturase, soybean  $\alpha'$  subunit of  $\beta$ -conglycinin (soy 7s, (Chen *et al.*, *Proc. Natl. Acad. Sci.*, 83:8560-8564 (1986))) and oleosin.

It may be advantageous to direct the localization of proteins conferring prenyltransferase to a particular subcellular compartment, for example, to the mitochondrion, endoplasmic

reticulum, vacuoles, chloroplast or other plastidic compartment. For example, where the genes of interest of the present invention will be targeted to plastids, such as chloroplasts, for expression, the constructs will also employ the use of sequences to direct the gene to the plastid. Such sequences are referred to herein as chloroplast transit peptides (CTP) or plastid transit peptides (PTP). In this manner, where the gene of interest is not directly inserted into the plastid, the expression construct will additionally contain a gene encoding a transit peptide to direct the gene of interest to the plastid. The chloroplast transit peptides may be derived from the gene of interest, or may be derived from a heterologous sequence having a CTP. Such transit peptides are known in the art. See, for example, Von Heijne *et al.* (1991) *Plant Mol. Biol. Rep.* 9:104-126; Clark *et al.* (1989) *J. Biol. Chem.* 264:17544-17550; della-Cioppa *et al.* (1987) *Plant Physiol.* 84:965-968; Romer *et al.* (1993) *Biochem. Biophys. Res Commun.* 196:1414-1421; and, Shah *et al.* (1986) *Science* 233:478-481.

Depending upon the intended use, the constructs may contain the nucleic acid sequence which encodes the entire prenyltransferase protein, or a portion thereof. For example, where antisense inhibition of a given prenyltransferase protein is desired, the entire prenyltransferase sequence is not required. Furthermore, where prenyltransferase sequences used in constructs are intended for use as probes, it may be advantageous to prepare constructs containing only a particular portion of a prenyltransferase encoding sequence, for example a sequence which is discovered to encode a highly conserved prenyltransferase region.

The skilled artisan will recognize that there are various methods for the inhibition of expression of endogenous sequences in a host cell. Such methods include, but are not limited to, antisense suppression (Smith, *et al.* (1988) *Nature* 334:724-726), co-suppression (Napoli, *et al.* (1989) *Plant Cell* 2:279-289), ribozymes (PCT Publication WO 97/10328), and combinations of sense and antisense Waterhouse, *et al.* (1998) *Proc. Natl. Acad. Sci. USA* 95:13959-13964. Methods for the suppression of endogenous sequences in a host cell typically employ the transcription or transcription and translation of at least a portion of the sequence to be suppressed. Such sequences may be homologous to coding as well as non-coding regions of the endogenous sequence.

Regulatory transcript termination regions may be provided in plant expression constructs of this invention as well. Transcript termination regions may be provided by the DNA sequence

encoding the prenyltransferase or a convenient transcription termination region derived from a different gene source, for example, the transcript termination region which is naturally associated with the transcript initiation region. The skilled artisan will recognize that any convenient transcript termination region which is capable of terminating transcription in a plant cell may be employed in the constructs of the present invention.

Alternatively, constructs may be prepared to direct the expression of the prenyltransferase sequences directly from the host plant cell plastid. Such constructs and methods are known in the art and are generally described, for example, in Svab, *et al.* (1990) *Proc. Natl. Acad. Sci. USA* 87:8526-8530 and Svab and Maliga (1993) *Proc. Natl. Acad. Sci. USA* 90:913-917 and in U.S. Patent Number 5,693,507.

The prenyltransferase constructs of the present invention can be used in transformation methods with additional constructs providing for the expression of other nucleic acid sequences encoding proteins involved in the production of tocopherols, or tocopherol precursors such as homogentisic acid and/or phytylpyrophosphate. Nucleic acid sequences encoding proteins involved in the production of homogentisic acid are known in the art, and include but not are limited to, 4-hydroxyphenylpyruvate dioxygenase (HPPD, EC 1.13.11.27) described for example, by Garcia, *et al.* ((1999) *Plant Physiol.* 119(4):1507-1516), mono or bifunctional tyrA (described for example by Xia, *et al.* (1992) *J. Gen Microbiol.* 138:1309-1316, and Hudson, *et al.* (1984) *J. Mol. Biol.* 180:1023-1051), Oxygenase, 4-hydroxyphenylpyruvate di- (9CI), 4-Hydroxyphenylpyruvate dioxygenase; p-Hydroxyphenylpyruvate dioxygenase; p-Hydroxyphenylpyruvate hydroxylase; p-Hydroxyphenylpyruvate oxidase; p-Hydroxyphenylpyruvic acid hydroxylase; p-Hydroxyphenylpyruvic hydroxylase; p-Hydroxyphenylpyruvic oxidase), 4-hydroxyphenylacetate, NAD(P)H: oxygen oxidoreductase (1-hydroxylating); 4-hydroxyphenylacetate 1-monooxygenase, and the like. In addition, constructs for the expression of nucleic acid sequences encoding proteins involved in the production of phytylpyrophosphate can also be employed with the prenyltransferase constructs of the present invention. Nucleic acid sequences encoding proteins involved in the production of phytylpyrophosphate are known in the art, and include, but are not limited to geranylgeranylpyrophosphate synthase (GGPPS), geranylgeranylpyrophosphate reductase (GGH), 1-deoxyxylulose-5-phosphate synthase, 1- deoxy-D-xylolose-5-phosphate

reductoisomerase, 4-diphosphocytidyl-2-C-methylerythritol synthase, isopentyl pyrophosphate isomerase.

The prenyltransferase sequences of the present invention find use in the preparation of transformation constructs having a second expression cassette for the expression of additional sequences involved in tocopherol biosynthesis. Additional tocopherol biosynthesis sequences of interest in the present invention include, but are not limited to gamma-tocopherol methyltransferase (Shintani, *et al.* (1998) *Science* 282(5396):2098-2100), tocopherol cyclase, and tocopherol methyltransferase.

A plant cell, tissue, organ, or plant into which the recombinant DNA constructs containing the expression constructs have been introduced is considered transformed, transfected, or transgenic. A transgenic or transformed cell or plant also includes progeny of the cell or plant and progeny produced from a breeding program employing such a transgenic plant as a parent in a cross and exhibiting an altered phenotype resulting from the presence of a prenyltransferase nucleic acid sequence.

Plant expression or transcription constructs having a prenyltransferase as the DNA sequence of interest for increased or decreased expression thereof may be employed with a wide variety of plant life, particularly, plant life involved in the production of vegetable oils for edible and industrial uses. Particularly preferred plants for use in the methods of the present invention include, but are not limited to: *Acacia*, alfalfa, aneth, apple, apricot, artichoke, arugula, asparagus, avocado, banana, barley, beans, beet, blackberry, blueberry, broccoli, brussels sprouts, cabbage, canola, cantaloupe, carrot, cassava, cauliflower, celery, cherry, chicory, cilantro, citrus, clementines, coffee, corn, cotton, cucumber, Douglas fir, eggplant, endive, escarole, eucalyptus, fennel, figs, garlic, gourd, grape, grapefruit, honey dew, jicama, kiwifruit, lettuce, leeks, lemon, lime, Loblolly pine, mango, melon, mushroom, nectarine, nut, oat, oil palm, oil seed rape, okra, onion, orange, an ornamental plant, papaya, parsley, pea, peach, peanut, pear, pepper, persimmon, pine, pineapple, plantain, plum, pomegranate, poplar, potato, pumpkin, quince, radiata pine, radicchio, radish, raspberry, rice, rye, sorghum, Southern pine, soybean, spinach, squash, strawberry, sugarbeet, sugarcane, sunflower, sweet potato, sweetgum, tangerine, tea, tobacco, tomato, triticale, turf, turnip, a vine, watermelon, wheat, yams, and zucchini.



Most especially preferred are temperate oilseed crops. Temperate oilseed crops of interest include, but are not limited to, rapeseed (Canola and High Erucic Acid varieties), sunflower, safflower, cotton, soybean, peanut, coconut and oil palms, and corn. Depending on the method for introducing the recombinant constructs into the host cell, other DNA sequences may be required. Importantly, this invention is applicable to dicotyledons and monocotyledons species alike and will be readily applicable to new and/or improved transformation and regulation techniques.

Of particular interest, is the use of prenyltransferase constructs in plants to produce plants or plant parts, including, but not limited to leaves, stems, roots, reproductive, and seed, with a modified content of tocopherols in plant parts having transformed plant cells.

For immunological screening, antibodies to the protein can be prepared by injecting rabbits or mice with the purified protein or portion thereof, such methods of preparing antibodies being well known to those in the art. Either monoclonal or polyclonal antibodies can be produced, although typically polyclonal antibodies are more useful for gene isolation. Western analysis may be conducted to determine that a related protein is present in a crude extract of the desired plant species, as determined by cross-reaction with the antibodies to the encoded proteins. When cross-reactivity is observed, genes encoding the related proteins are isolated by screening expression libraries representing the desired plant species. Expression libraries can be constructed in a variety of commercially available vectors, including lambda gt11, as described in Sambrook, *et al.* (*Molecular Cloning: A Laboratory Manual*, Second Edition (1989) Cold Spring Harbor Laboratory, Cold Spring Harbor, New York).

To confirm the activity and specificity of the proteins encoded by the identified nucleic acid sequences as prenyltransferase enzymes, *in vitro* assays are performed in insect cell cultures using baculovirus expression systems. Such baculovirus expression systems are known in the art and are described by Lee, *et al.* U.S. Patent Number 5,348,886, the entirety of which is herein incorporated by reference.

In addition, other expression constructs may be prepared to assay for protein activity utilizing different expression systems. Such expression constructs are transformed into yeast or prokaryotic host and assayed for prenyltransferase activity. Such expression systems are known in the art and are readily available through commercial sources.

In addition to the sequences described in the present invention, DNA coding sequences useful in the present invention can be derived from algae, fungi, bacteria, mammalian sources, plants, etc. Homology searches in existing databases using signature sequences corresponding to conserved nucleotide and amino acid sequences of prenyltransferase can be employed to isolate equivalent, related genes from other sources such as plants and microorganisms. Searches in EST databases can also be employed. Furthermore, the use of DNA sequences encoding enzymes functionally enzymatically equivalent to those disclosed herein, wherein such DNA sequences are degenerate equivalents of the nucleic acid sequences disclosed herein in accordance with the degeneracy of the genetic code, is also encompassed by the present invention. Demonstration of the functionality of coding sequences identified by any of these methods can be carried out by complementation of mutants of appropriate organisms, such as *Synechocystis*, *Shewanella*, yeast, *Pseudomonas*, *Rhodobacteria*, etc., that lack specific biochemical reactions, or that have been mutated. The sequences of the DNA coding regions can be optimized by gene resynthesis, based on codon usage, for maximum expression in particular hosts.

For the alteration of tocopherol production in a host cell, a second expression construct can be used in accordance with the present invention. For example, the prenyltransferase expression construct can be introduced into a host cell in conjunction with a second expression construct having a nucleotide sequence for a protein involved in tocopherol biosynthesis.

The method of transformation in obtaining such transgenic plants is not critical to the instant invention, and various methods of plant transformation are currently available. Furthermore, as newer methods become available to transform crops, they may also be directly applied hereunder. For example, many plant species naturally susceptible to *Agrobacterium* infection may be successfully transformed via tripartite or binary vector methods of *Agrobacterium* mediated transformation. In many instances, it will be desirable to have the construct bordered on one or both sides by T-DNA, particularly having the left and right borders, more particularly the right border. This is particularly useful when the construct uses *A. tumefaciens* or *A. rhizogenes* as a mode for transformation, although the T-DNA borders may find use with other modes of transformation. In addition, techniques of microinjection, DNA

particle bombardment, and electroporation have been developed which allow for the transformation of various monocot and dicot plant species.

Normally, included with the DNA construct will be a structural gene having the necessary regulatory regions for expression in a host and providing for selection of transformant cells. The gene may provide for resistance to a cytotoxic agent, e.g. antibiotic, heavy metal, toxin, etc., complementation providing prototrophy to an auxotrophic host, viral immunity or the like. Depending upon the number of different host species the expression construct or components thereof are introduced, one or more markers may be employed, where different conditions for selection are used for the different hosts.

Where *Agrobacterium* is used for plant cell transformation, a vector may be used which may be introduced into the *Agrobacterium* host for homologous recombination with T-DNA or the Ti- or Ri-plasmid present in the *Agrobacterium* host. The Ti- or Ri-plasmid containing the T-DNA for recombination may be armed (capable of causing gall formation) or disarmed (incapable of causing gall formation), the latter being permissible, so long as the *vir* genes are present in the transformed *Agrobacterium* host. The armed plasmid can give a mixture of normal plant cells and gall.

In some instances where *Agrobacterium* is used as the vehicle for transforming host plant cells, the expression or transcription construct bordered by the T-DNA border region(s) will be inserted into a broad host range vector capable of replication in *E. coli* and *Agrobacterium*, there being broad host range vectors described in the literature. Commonly used is pRK2 or derivatives thereof. See, for example, Ditta, *et al.*, (*Proc. Nat. Acad. Sci., U.S.A.* (1980) 77:7347-7351) and EPA 0 120 515, which are incorporated herein by reference. Alternatively, one may insert the sequences to be expressed in plant cells into a vector containing separate replication sequences, one of which stabilizes the vector in *E. coli*, and the other in *Agrobacterium*. See, for example, McBride, *et al.* (*Plant Mol. Biol.* (1990) 14:269-276), wherein the pRiHRI (Jouanin, *et al.*, *Mol. Gen. Genet.* (1985) 201:370-374) origin of replication is utilized and provides for added stability of the plant expression vectors in host *Agrobacterium* cells.

Included with the expression construct and the T-DNA will be one or more markers, which allow for selection of transformed *Agrobacterium* and transformed plant cells. A

number of markers have been developed for use with plant cells, such as resistance to chloramphenicol, kanamycin, the aminoglycoside G418, hygromycin, or the like. The particular marker employed is not essential to this invention, one or another marker being preferred depending on the particular host and the manner of construction.

5 For transformation of plant cells using *Agrobacterium*, explants may be combined and incubated with the transformed *Agrobacterium* for sufficient time for transformation, the bacteria killed, and the plant cells cultured in an appropriate selective medium. Once callus forms, shoot formation can be encouraged by employing the appropriate plant hormones in accordance with known methods and the shoots transferred to rooting medium for regeneration of plants. The  
10 plants may then be grown to seed and the seed used to establish repetitive generations and for isolation of vegetable oils.

There are several possible ways to obtain the plant cells of this invention which contain multiple expression constructs. Any means for producing a plant comprising a construct having a DNA sequence encoding the expression construct of the present invention, and at least one other construct having another DNA sequence encoding an enzyme are encompassed by the present invention. For example, the expression construct can be used to transform a plant at the same time as the second construct either by inclusion of both expression constructs in a single transformation vector or by using separate vectors, each of which express desired genes. The second construct can be introduced into a plant which has already been transformed with the prenyltransferase expression construct, or alternatively, transformed plants, one expressing the prenyltransferase construct and one expressing the second construct, can be crossed to bring the constructs together in the same plant.

The nucleic acid sequences of the present invention can be used in constructs to provide for the expression of the sequence in a variety of host cells, both prokaryotic eukaryotic. Host  
25 cells of the present invention preferably include monocotyledenous and dicotyledenous plant cells.

In general, the skilled artisan is familiar with the standard resource materials which describe specific conditions and procedures for the construction, manipulation and isolation of macromolecules (e.g., DNA molecules, plasmids, etc.), generation of recombinant organisms and  
30 the screening and isolating of clones, (see for example, Sambrook *et al.*, *Molecular Cloning: A*

*Laboratory Manual*, Cold Spring Harbor Press (1989); Maliga *et al.*, *Methods in Plant Molecular Biology*, Cold Spring Harbor Press (1995), the entirety of which is herein incorporated by reference; Birren *et al.*, *Genome Analysis: Analyzing DNA*, 1, Cold Spring Harbor, New York, the entirety of which is herein incorporated by reference).

5           Methods for the expression of sequences in insect host cells are known in the art. Baculovirus expression vectors are recombinant insect viruses in which the coding sequence for a chosen foreign gene has been inserted behind a baculovirus promoter in place of the viral gene, e.g., polyhedrin (Smith and Summers, U.S. Pat. No., 4,745,051, the entirety of which is incorporated herein by reference). Baculovirus expression vectors are known in the art, and are  
10           described for example in Doerfler, *Curr. Top. Microbiol. Immunol.* 131:51-68 (1968); Luckow and Summers, *Bio/Technology* 6:47-55 (1988a); Miller, *Annual Review of Microbiol.* 42:177-199 (1988); Summers, *Curr. Comm. Molecular Biology*, Cold Spring Harbor Press, Cold Spring Harbor, N.Y. (1988); Summers and Smith, *A Manual of Methods for Baculovirus Vectors and Insect Cell Culture Procedures*, Texas Ag. Exper. Station Bulletin No. 1555 (1988), the entireties of which is herein incorporated by reference)

          Methods for the expression of a nucleic acid sequence of interest in a fungal host cell are known in the art. The fungal host cell may, for example, be a yeast cell or a filamentous fungal cell. Methods for the expression of DNA sequences of interest in yeast cells are generally described in "Guide to yeast genetics and molecular biology", Guthrie and Fink, eds. *Methods in enzymology*, Academic Press, Inc. Vol 194 (1991) and *Gene expression technology*, Goeddel ed, *Methods in Enzymology*, Academic Press, Inc., Vol 185 (1991).

          Mammalian cell lines available as hosts for expression are known in the art and include many immortalized cell lines available from the American Type Culture Collection (ATCC, Manassas, VA), such as HeLa cells, Chinese hamster ovary (CHO) cells, baby hamster kidney  
25           (BHK) cells and a number of other cell lines. Suitable promoters for mammalian cells are also known in the art and include, but are not limited to, viral promoters such as that from Simian Virus 40 (SV40) (Fiers *et al.*, *Nature* 273:113 (1978), the entirety of which is herein incorporated by reference), Rous sarcoma virus (RSV), adenovirus (ADV) and bovine papilloma virus (BPV). Mammalian cells may also require terminator sequences and poly-A addition sequences.

30           Enhancer sequences which increase expression may also be included and sequences which

promote amplification of the gene may also be desirable (for example methotrexate resistance genes).

Vectors suitable for replication in mammalian cells are well known in the art, and may include viral replicons, or sequences which insure integration of the appropriate sequences encoding epitopes into the host genome. Plasmid vectors that greatly facilitate the construction of recombinant viruses have been described (*see, for example, Mackett et al, J Virol. 49:857* (1984); Chakrabarti *et al., Mol. Cell. Biol. 5:3403* (1985); Moss, In: *Gene Transfer Vectors For Mammalian Cells* (Miller and Calos, eds., Cold Spring Harbor Laboratory, N.Y., p. 10, (1987); all of which are herein incorporated by reference in their entirety).

The invention now being generally described, it will be more readily understood by reference to the following examples which are included for purposes of illustration only and are not intended to limit the present invention.

## EXAMPLES

### Example 1: Identification of Prenyltransferase Sequences

PSI-BLAST (Altschul, *et al. (1997) Nuc Acid Res 25:3389-3402*) profiles were generated for both the straight chain and aromatic classes of prenyltransferases. To generate the straight chain profile, a prenyl-transferase from *Porphyra purpurea* (Genbank accession 1709766) was used as a query against the NCBI non-redundant protein database. The *E. coli* enzyme involved in the formation of ubiquinone, ubiA (genbank accession 1790473) was used as a starting sequence to generate the aromatic prenyltransferase profile. These profiles were used to search public and proprietary DNA and protein data bases. In *Arabidopsis* seven putative prenyltransferases of the straight-chain class were identified, ATPT1, (SEQ ID NO:9), ATPT7 (SEQ ID NO:10), ATPT8 (SEQ ID NO:11), ATPT9 (SEQ ID NO:13), ATPT10 (SEQ ID NO:14), ATPT11 (SEQ ID NO:15), and ATPT12 (SEQ ID NO:16) and five were identified of the aromatic class, ATPT2 (SEQ ID NO:1), ATPT3 (SEQ ID NO:3), ATPT4 (SEQ ID NO:5), ATPT5 (SEQ ID NO:7), ATPT6 (SEQ ID NO:8). Additional prenyltransferase sequences from

other plants related to the aromatic class of prenyltransferases, such as soy (SEQ IDNOs: 19-23, the deduced amino acid sequence of SEQ ID NO:23 is provided in SEQ ID NO:24) and maize (SEQ ID NOs:25-29, and 31) are also identified. The deduced amino acid sequence of ZMPT5 (SEQ ID NO:29) is provided in SEQ ID NO:30.

5 Searches are performed on a Silicon Graphics Unix computer using additional Bioaccelerator hardware and GenWeb software supplied by Compugen Ltd. This software and hardware enables the use of the Smith-Waterman algorithm in searching DNA and protein databases using profiles as queries. The program used to query protein databases is profilesearch. This is a search where the query is not a single sequence but a profile based on a multiple  
10 alignment of amino acid or nucleic acid sequences. The profile is used to query a sequence data set, i.e., a sequence database. The profile contains all the pertinent information for scoring each position in a sequence, in effect replacing the "scoring matrix" used for the standard query searches. The program used to query nucleotide databases with a protein profile is tprofilesearch. Tprofilesearch searches nucleic acid databases using an amino acid profile query. As the search is running, sequences in the database are translated to amino acid sequences in six reading frames. The output file for tprofilesearch is identical to the output file for profilesearch except for an additional column that indicates the frame in which the best alignment occurred.

The Smith-Waterman algorithm, (Smith and Waterman (1981) *supra*), is used to search for similarities between one sequence from the query and a group of sequences contained in the database. E score values as well as other sequence information, such as conserved peptide sequences are used to identify related sequences.

To obtain the entire coding region corresponding to the *Arabidopsis* prenyltransferase sequences, synthetic oligo-nucleotide primers are designed to amplify the 5' and 3' ends of partial cDNA clones containing prenyltransferase sequences. Primers are designed according to  
25 the respective *Arabidopsis* prenyltransferase sequences and used in Rapid Amplification of cDNA Ends (RACE) reactions (Frohman *et al.* (1988) *Proc. Natl. Acad. Sci. USA* 85:8998-9002) using the Marathon cDNA amplification kit (Clontech Laboratories Inc, Palo Alto, CA).

Additional BLAST searches are performed using the ATPT2 sequence, a sequence in the class of aromatic prenyl transferases. Additional sequences are identified in soybean libraries

that are similar to the ATPT2 sequence. The additional soybean sequence demonstrates 80% identity and 91% similarity at the amino acid sequence.

Amino acid sequence alignments between ATPT2 (SEQ ID NO:2), ATPT3 (SEQ ID NO:4), ATPT4 (SEQ ID NO:6), ATPT8 (SEQ ID NO:12), and ATPT12 (SEQ ID NO:17) are performed using ClustalW (Figure 1), and the percent identity and similarities are provided in Table 1 below.

**Table 1:**

		ATPT2	ATPT3	ATPT4	ATPT8	ATPT12
ATPT2	% Identity		12	13	11	15
	% similar		25	25	22	32
	% Gap		17	20	20	9
ATPT3	% Identity			12	6	22
	% similar			29	16	38
	% Gap			20	24	14
ATPT4	% Identity				9	14
	% similar				18	29
	% Gap				26	19
ATPT8	% Identity					7
	% similar					19
	% Gap					20
ATPT12	% Identity					
	% similar					
	% Gap					

**Example 2: Preparation of Expression Constructs**

A plasmid containing the napin cassette derived from pCGN3223 (described in USPN 5,639,790, the entirety of which is incorporated herein by reference) was modified to make it



more useful for cloning large DNA fragments containing multiple restriction sites, and to allow the cloning of multiple napin fusion genes into plant binary transformation vectors. An adapter comprised of the self annealed oligonucleotide of sequence

CGCGATTTAAATGGCGCGCCCTGCAGGCGGCCGCTGCAGGGCGCGCCATTTAAAT

(SEQ ID NO:40) was ligated into the cloning vector pBC SK+ (Stratagene) after digestion with the restriction endonuclease BssHII to construct vector pCGN7765. Plasmids pCGN3223 and pCGN7765 were digested with NotI and ligated together. The resultant vector, pCGN7770, contains the pCGN7765 backbone with the napin seed specific expression cassette from pCGN3223.

The cloning cassette, pCGN7787, essentially the same regulatory elements as pCGN7770, with the exception of the napin regulatory regions of pCGN7770 have been replaced with the double CAMV 35S promoter and the tml polyadenylation and transcriptional termination region.

A binary vector for plant transformation, pCGN5139, was constructed from pCGN1558 (McBride and Summerfelt, (1990) Plant Molecular Biology, 14:269-276). The polylinker of pCGN1558 was replaced as a HindIII/Asp718 fragment with a polylinker containing unique restriction endonuclease sites, AscI, PacI, XbaI, SmaI, BamHI, and NotI. The Asp718 and HindIII restriction endonuclease sites are retained in pCGN5139.

A series of turbo binary vectors are constructed to allow for the rapid cloning of DNA sequences into binary vectors containing transcriptional initiation regions (promoters) and transcriptional termination regions.

The plasmid pCGN8618 was constructed by ligating oligonucleotides 5'-TCGAGGATCCGCGGCCGCAAGCTTCCTGCAGG-3' (SEQ ID NO:41) and 5'-TCGACCTGCAGGAAGCTTGCGGCCGCGGATCC-3' (SEQ ID NO:42) into SalI/XhoI-digested pCGN7770. A fragment containing the napin promoter, polylinker and napin 3' region was excised from pCGN8618 by digestion with Asp718I; the fragment was blunt-ended by filling in the 5' overhangs with Klenow fragment then ligated into pCGN5139 that had been digested with Asp718I and HindIII and blunt-ended by filling in the 5' overhangs with Klenow fragment. A plasmid containing the insert oriented so that the napin promoter was closest to the blunted Asp718I site of pCGN5139 and the napin 3' was closest to the blunted HindIII site was subjected

to sequence analysis to confirm both the insert orientation and the integrity of cloning junctions. The resulting plasmid was designated pCGN8622.

The plasmid pCGN8619 was constructed by ligating oligonucleotides 5'-TCGACCTGCAGGAAGCTTGCGGCCGCGGATCC -3' (SEQ ID NO:43) and 5'-TCGAGGATCCGCGGCCGCAAGCTTCCTGCAGG-3' (SEQ ID NO:44) into SalI/XhoI-digested pCGN7770. A fragment containing the napin promoter, polylinker and napin 3' region was removed from pCGN8619 by digestion with Asp718I; the fragment was blunt-ended by filling in the 5' overhangs with Klenow fragment then ligated into pCGN5139 that had been digested with Asp718I and HindIII and blunt-ended by filling in the 5' overhangs with Klenow fragment. A plasmid containing the insert oriented so that the napin promoter was closest to the blunted Asp718I site of pCGN5139 and the napin 3' was closest to the blunted HindIII site was subjected to sequence analysis to confirm both the insert orientation and the integrity of cloning junctions. The resulting plasmid was designated pCGN8623.

The plasmid pCGN8620 was constructed by ligating oligonucleotides 5'-TCGAGGATCCGCGGCCGCAAGCTTCCTGCAGGAGCT -3' (SEQ ID NO:45) and 5'-CCTGCAGGAAGCTTGCGGCCGCGGATCC-3' (SEQ ID NO:46) into SalI/SacI-digested pCGN7787. A fragment containing the d35S promoter, polylinker and tml 3' region was removed from pCGN8620 by complete digestion with Asp718I and partial digestion with NotI. The fragment was blunt-ended by filling in the 5' overhangs with Klenow fragment then ligated into pCGN5139 that had been digested with Asp718I and HindIII and blunt-ended by filling in the 5' overhangs with Klenow fragment. A plasmid containing the insert oriented so that the d35S promoter was closest to the blunted Asp718I site of pCGN5139 and the tml 3' was closest to the blunted HindIII site was subjected to sequence analysis to confirm both the insert orientation and the integrity of cloning junctions. The resulting plasmid was designated pCGN8624.

The plasmid pCGN8621 was constructed by ligating oligonucleotides 5'-TCGACCTGCAGGAAGCTTGCGGCCGCGGATCCAGCT -3' (SEQ ID NO:47) and 5'-GGATCCGCGGCCGCAAGCTTCCTGCAGG-3' (SEQ ID NO:48) into SalI/SacI-digested pCGN7787. A fragment containing the d35S promoter, polylinker and tml 3' region was removed from pCGN8621 by complete digestion with Asp718I and partial digestion with NotI.

The fragment was blunt-ended by filling in the 5' overhangs with Klenow fragment then ligated into pCGN5139 that had been digested with Asp718I and HindIII and blunt-ended by filling in the 5' overhangs with Klenow fragment. A plasmid containing the insert oriented so that the d35S promoter was closest to the blunted Asp718I site of pCGN5139 and the tml 3' was closest to the blunted HindIII site was subjected to sequence analysis to confirm both the insert orientation and the integrity of cloning junctions. The resulting plasmid was designated pCGN8625.

The plasmid construct pCGN8640 is a modification of pCGN8624 described above. A 938bp PstI fragment isolated from transposon Tn7 which encodes bacterial spectinomycin and streptomycin resistance (Fling et al. (1985), *Nucleic Acids Research* 13(19):7095-7106), a determinant for E. coli and Agrobacterium selection, was blunt ended with Pfu polymerase. The blunt ended fragment was ligated into pCGN8624 that had been digested with SpeI and blunt ended with Pfu polymerase. The region containing the PstI fragment was sequenced to confirm both the insert orientation and the integrity of cloning junctions.

The spectinomycin resistance marker was introduced into pCGN8622 and pCGN8623 as follows. A 7.7 Kbp AvrII-SnaBI fragment from pCGN8640 was ligated to a 10.9 Kbp AvrII-SnaBI fragment from pCGN8623 or pCGN8622, described above. The resulting plasmids were pCGN8641 and pCGN8643, respectively.

The plasmid pCGN8644 was constructed by ligating oligonucleotides 5'-GATCACCTGCAGGAAGCTTGC GGCCGCGGATCCAATGCA-3' (SEQ ID NO:49) and 5'-TTGGATCCGCGGCCGCAAGCTTCCTGCAGGT-3' (SEQ ID NO:50) into BamHI-PstI digested pCGN8640.

Synthetic oligonucleotides were designed for use in Polymerase Chain Reactions (PCR) to amplify the coding sequences of ATPT2, ATPT3, ATPT4, ATPT8, and ATPT12 for the preparation of expression constructs and are provided in Table 2 below.

Table 2:

Name	Restriction Site	Sequence	SEQ ID NO:
ATPT2	5' NotI	GGATCCGCGGCCGCAACAATGGAGTC TCTGCTCTCTAGTTCT	51
ATPT2	3' SseI	GGATCCTGCAGGTCACTTCAAAAAA GGTAACAGCAAGT	52
ATPT3	5' NotI	GGATCCGCGGCCGCAACAATGGCGTT	53

		TTTTGGGCTCTCCCGTGTTT	
ATPT3	3' SseI	GGATCCTGCAGGTTATTGAAAACCT CTTCCAAGTACAAC	54
ATPT4	5' NotI	GGATCCGCGGCCGCACAATGTGGCG AAGATCTGTTGTT	55
ATPT4	3' SseI	GGATCCTGCAGGTCATGGAGAGTAG AAGGAAGGAGCT	56
ATPT8	5' NotI	GGATCCGCGGCCGCACAATGGTACT TGCCGAGGTTCCAAAGCTTGCCTCT	57
ATPT8	3' SseI	GGATCCTGCAGGTCACCTGTTTCTG GTGATGACTCTAT	58
ATPT12	5' NotI	GGATCCGCGGCCGCACAATGACTTC GATTCTCAACACT	59
ATPT12	3' SseI	GGATCCTGCAGGTCAGTGTTGCGAT GCTAATGCCGT	60

The coding sequences of ATPT2, ATPT3, ATPT4, ATPT8, and ATPT12 were all amplified using the respective PCR primers shown in Table 2 above and cloned into the TopoTA vector (Invitrogen). Constructs containing the respective prenyltransferase sequences were digested with NotI and Sse8387I and cloned into the turbobinary vectors described above.

The sequence encoding ATPT2 prenyltransferase was cloned in the sense orientation into pCGN8640 to produce the plant transformation construct pCGN10800 (Figure 2). The ATPT2 sequence is under control of the 35S promoter.

The ATPT2 sequence was also cloned in the antisense orientation into the construct pCGN8641 to create pCGN10801 (Figure 3). This construct provides for the antisense expression of the ATPT2 sequence from the napin promoter.

The ATPT2 coding sequence was also cloned in the antisense orientation into the vector pCGN8643 to create the plant transformation construct pCGN10802

The ATPT2 coding sequence was also cloned in the antisense orientation into the vector pCGN8644 to create the plant transformation construct pCGN10803 (Figure 4).

The ATPT4 coding sequence was cloned into the vector pCGN864 to create the plant transformation construct pCGN10806 (Figure 5). The ATPT2 coding sequence was cloned into the vector pCGN864 to create the plant transformation construct pCGN10807 (Figure 6). The ATPT3 coding sequence was cloned into the vector pCGN864 to create the plant transformation construct pCGN10808 (Figure 7). The ATPT3 coding sequence was cloned in the sense orientation into the

vector pCGN8640 to create the plant transformation construct pCGN10809 (Figure 8). The ATPT3 coding sequence was cloned in the antisense orientation into the vector pCGN8641 to create the plant transformation construct pCGN10810 (Figure 9). The ATPT3 coding sequence was cloned into the vector pCGN8643 to create the plant transformation construct pCGN10811 (Figure 10). The ATPT3 coding sequence was cloned into the vector pCGN8640 to create the plant transformation construct pCGN10812 (Figure 11). The ATPT4 coding sequence was cloned into the vector pCGN8640 to create the plant transformation construct pCGN10813 (Figure 12). The ATPT4 coding sequence was cloned into the vector pCGN8643 to create the plant transformation construct pCGN10814 (Figure 13). The ATPT4 coding sequence was cloned into the vector pCGN8641 to create the plant transformation construct pCGN10815 (Figure 14). The ATPT4 coding sequence was cloned in the antisense orientation into the vector pCGN8644 to create the plant transformation construct pCGN10816 (Figure 15). The ATPT2 coding sequence was cloned into the vector pCGN???? to create the plant transformation construct pCGN10817 (Figure 16). The ATPT8 coding sequence was cloned in the sense orientation into the vector pCGN8643 to create the plant transformation construct pCGN10819 (Figure 17). The ATPT12 coding sequence was cloned into the vector pCGN8644 to create the plant transformation construct pCGN10824 (Figure 18). The ATPT12 coding sequence was cloned into the vector pCGN8641 to create the plant transformation construct pCGN10825 (Figure 19). The ATPT8 coding sequence was cloned into the vector pCGN8644 to create the plant transformation construct pCGN10826 (Figure 20).

### Example 3: Plant Transformation

Transgenic *Brassica* plants are obtained by *Agrobacterium*-mediated transformation as described by Radke *et al.* (*Theor. Appl. Genet.* (1988) 75:685-694; *Plant Cell Reports* (1992) 11:499-505). Transgenic *Arabidopsis thaliana* plants may be obtained by *Agrobacterium*-mediated transformation as described by Valverkens *et al.*, (*Proc. Nat. Acad. Sci.* (1988) 85:5536-5540), or as described by Bent *et al.* ((1994), *Science* 265:1856-1860), or Bechtold *et al.* ((1993), *C.R.Acad.Sci, Life Sciences* 316:1194-1199). Other plant species may be similarly transformed using related techniques.

Alternatively, microprojectile bombardment methods, such as described by Klein *et al.* (*Bio/Technology* 10:286-291) may also be used to obtain nuclear transformed plants.

5 **Example 4: Identification of Additional Prenyltransferases**

A PSI-Blast profile generated using the *E. coli* ubiA (genbank accession 1790473) sequence was used to analyze the *Synechocystis* genome. This analysis identified 5 open reading frames (ORFs) in the *Synechocystis* genome that were potentially prenyltransferases; slr0926 (annotated as ubiA (4-hydroxybenzoate-octaprenyl transferase, SEQ ID NO:32), slr1899 (annotated as ctaB (cytochrome c oxidase folding protein, SEQ ID NO:33), slr0056 (annotated as g4 (chlorophyll synthase 33 kd subunit, SEQ ID NO:34), slr1518 (annotated as menA (menaquinone biosynthesis protein, SEQ ID NO:35), and slr1736 (annotated as a hypothetical protein of unknown function (SEQ ID NO:36).

To determine the functionality of these ORFs and their involvement, if any, in the biosynthesis of Tocopherols, knockouts constructs were made to disrupt the ORF identified in *Synechocystis*.

Synthetic oligos were designed to amplify regions from the 5' (5'-TAATGTGTACATTGTCGGCCTC (17365') (SEQ ID NO:61) and 5'-GCAATGTAACATCAGAGATTTTGAGACACAACGTGGCTTTCCACAATTCCCCGCACC GTC (1736kanpr1)) (SEQ ID NO:62) and 3' (5'-AGGCTAATAAGCACAAATGGGA (17363') (SEQ ID NO:63) and 5'-GGTATGAGTCAGCAACACCTTCTTCACGAGGCAGACCTCAGC GGAATTGGTTTtaggttATCCC (1736kanpr2)) (SEQ ID NO:64) ends of the slr1736 ORF.

The 1736kanpr1 and 1736kanpr2 oligos contained 20 bp of homology to the slr1736 ORF with an additional 40 bp of sequence homology to the ends of the kanamycin resistance cassette.

Separate PCR steps were completed with these oligos and the products were gel purified and combined with the kanamycin resistance gene from puc4K (Pharmacia) that had been digested with *HincII* and gel purified away from the vector backbone. The combined fragments were allowed to assemble without oligos under the following conditions: 94°C for 1 min, 55°C for 1 min, 72°C for 1 min plus 5 seconds per cycle for 40 cycles using pfu polymerase in 100ul

reaction volume (Zhao, H and Arnold (1997) *Nucleic Acids Res.* 25(6):1307-1308). One microliter or five microliters of this assembly reaction was then amplified using 5' and 3' oligos nested within the ends of the ORF fragment, so that the resulting product contained 100-200 bp of the 5' end of the *Synechocystis* gene to be knocked out, the kanamycin resistance cassette, and 100-200 bp of the 3' end of the gene to be knocked out. This PCR product was then cloned into the vector pGemT easy (Promega) to create the construct pMON21681 and used for *Synechocystis* transformation.

Primers were also synthesized for the preparation of *Synechocystis* knockout constructs for the other sequences using the same method as described above, with the following primers.

The *ubiA* 5' sequence was amplified using the primers 5'- GGATCCATGGTT GCCCAAACCCCATC (SEQ ID NO:65) and 5'- GCAATGTAACATCAGAGA TTTTGAGACACAACG TGGCTTTGGGTAAGCAACAATGACCGGC (SEQ ID NO:66). The 3' region was amplified using the synthetic oligonucleotide primers 5'- GAATTCTCAAAGCCAGCCCAGTAAC (SEQ ID NO:67) and 5'-GGTATGAGTC AGCAACACCTTCTTCACGAGGCAGACCTCAGCGGGTGCGAAAAGGGTTTTCCC (SEQ ID NO:68). The amplification products were combined with the kanamycin resistance gene from puc4K (Pharmacia) that had been digested with *HincII* and gel purified away from the vector backbone. The annealed fragment was amplified using 5' and 3' oligos nested within the ends of the ORF fragment (5'- CCAGTGGTTTAGGCTGTGTGGTC (SEQ ID NO:69) and 5'- CTGAGTTGGATGTATTGGATC (SEQ ID NO:70)), so that the resulting product contained 100-200 bp of the 5' end of the *Synechocystis* gene to be knocked out, the kanamycin resistance cassette, and 100-200 bp of the 3' end of the gene to be knocked out. This PCR product was then cloned into the vector pGemT easy (Promega) to create the construct pMON21682 and used for *Synechocystis* transformation.

Primers were also synthesized for the preparation of *Synechocystis* knockout constructs for the other sequences using the same method as described above, with the following primers. The *sl11899* 5' sequence was amplified using the primers 5'- GGATCCATGGTTACTT CGACAAAAATCC (SEQ ID NO:71) and 5'- GCAATGTAACATCAGAG ATTTTGAGACACAACGTGGCTTTGCTAGGCAACCGCTTAGTAC (SEQ ID NO:72). The 3' region was amplified using the synthetic oligonucleotide primers 5'-

GAATTCTTAACCCAACAGTAAAGTTCCC (SEQ ID NO:73) and 5'-GGTATGAGTCAGC  
AACACCTTCTTCACGAGGCAGACCTCAGCGCCGGCATTGTCTTTTACATG (SEQ ID  
NO:74). The amplification products were combined with the kanamycin resistance gene from  
puc4K (Pharmacia) that had been digested with *HincII* and gel purified away from the vector  
backbone. The annealed fragment was amplified using 5' and 3' oligos nested within the ends of  
the ORF fragment (5'-GGAACCCTTGCAGCCGCTTC (SEQ ID NO:75)  
and 5'-GTATGCCCAACTGGTGCAGAGG (SEQ ID NO:76)), so that the resulting product  
contained 100-200 bp of the 5' end of the *Synechocystis* gene to be knocked out, the kanamycin  
resistance cassette, and 100-200 bp of the 3' end of the gene to be knocked out. This PCR  
product was then cloned into the vector pGemT easy (Promega) to create the construct  
pMON21679 and used for *Synechocystis* transformation.

Primers were also synthesized for the preparation of *Synechocystis* knockout constructs  
for the other sequences using the same method as described above, with the following primers.  
The slr0056 5' sequence was amplified using the primers 5'-  
GGATCCATGTCTGACACACAAAATACCG (SEQ ID NO:77) and 5'-  
GCAATGTAACATCAGAGATTTTGAGACACAACGTGGCTTTCGCCAATACCAGCCACC  
AACAG (SEQ ID NO:78). The 3' region was amplified using the synthetic oligonucleotide  
primers 5'-GAATTCTCAAATCCCCGCATGGCCTAG (SEQ ID NO:79) and 5'-  
GGTATGAGTCAGCAACACCTTCTTCACGAGGCAGACCTCAGCGGCCTACGGCTTGGA  
CGTGTGGG (SEQ ID NO:80). The amplification products were combined with the kanamycin  
resistance gene from puc4K (Pharmacia) that had been digested with *HincII* and gel purified  
away from the vector backbone. The annealed fragment was amplified using 5' and 3' oligos  
nested within the ends of the ORF fragment (5'-CACTTGGATTCCCCTGATCTG (SEQ ID  
NO:81) and 5'-GCAATACCCGCTTGGAACG (SEQ ID NO:82)), so that the resulting  
product contained 100-200 bp of the 5' end of the *Synechocystis* gene to be knocked out, the  
kanamycin resistance cassette, and 100-200 bp of the 3' end of the gene to be knocked out. This  
PCR product was then cloned into the vector pGemT easy (Promega) to create the construct  
pMON21677 and used for *Synechocystis* transformation.

Primers were also synthesized for the preparation of *Synechocystis* knockout constructs  
for the other sequences using the same method as described above, with the following primers.



The slr1518 5' sequence was amplified using the primers 5'- GGATCCATGACCGAAT  
CTTCGCCCCCTAGC (SEQ ID NO:83) and 5'-GCAATGTAACATCAGAGATTTTGA  
GACACAACGTGGC TTTCAATCCTAGGTAGCCGAGGCG (SEQ ID NO:84). The 3' region  
was amplified using the synthetic oligonucleotide primers 5'- GAATTCTTAGCCCAGGCC  
5 AGCCAGCC (SEQ ID NO:85) and 5'- GGTATGAGTCAGCAACACCTTCTTCACGA  
GGCAGACCTCAGCGGGGAATTGATTTGTTTAATTACC (SEQ ID NO:86). The  
amplification products were combined with the kanamycin resistance gene from puc4K  
(Pharmacia) that had been digested with *HincII* and gel purified away from the vector backbone.  
The annealed fragment was amplified using 5' and 3' oligos nested within the ends of the ORF  
10 fragment (5'- GCGATCGCCATTATCGCTTGG (SEQ ID NO:87) and 5'-  
GCAGACTGGCAATTATCAGTAACG (SEQ ID NO:88)), so that the resulting product  
contained 100-200 bp of the 5' end of the *Synechocystis* gene to be knocked out, the kanamycin  
resistance cassette, and 100-200 bp of the 3' end of the gene to be knocked out. This PCR  
product was then cloned into the vector pGemT easy (Promega) to create the construct  
pMON21680 and used for *Synechocystis* transformation.

#### B. Transformation of *Synechocystis*

Cells of *Synechocystis* 6803 were grown to a density of approximately  $2 \times 10^8$  cells per ml  
and harvested by centrifugation. The cell pellet was re-suspended in fresh BG-11 medium  
(ATCC Medium 616) at a density of  $1 \times 10^9$  cells per ml and used immediately for transformation.  
One-hundred microliters of these cells were mixed with 5 ul of mini prep DNA and incubated  
with light at 30C for 4 hours. This mixture was then plated onto nylon filters resting on BG-11  
agar supplemented with TES pH8 and allowed to grow for 12-18 hours. The filters were then  
transferred to BG-11 agar + TES + 5ug/ml kanamycin and allowed to grow until colonies  
25 appeared within 7-10 days (Packer and Glazer, 1988). Colonies were then picked into BG-11  
liquid media containing 5 ug/ml kanamycin and allowed to grow for 5 days. These cells were  
then transferred to Bg-11 media containing 10ug/ml kanamycin and allowed to grow for 5 days  
and then transferred to Bg-11 + kanamycin at 25ug/ml and allowed to grow for 5 days. Cells  
were then harvested for PCR analysis to determine the presence of a disrupted ORF and also for  
30 HPLC analysis to determine if the disruption had any effect on tocopherol levels.

PCR analysis of the *Synechocystis* isolates for slr1736 and sl11899 showed complete segregation of the mutant genome, meaning no copies of the wild type genome could be detected in these strains. This suggests that function of the native gene is not essential for cell function. HPLC analysis of these same isolates showed that the sl11899 strain had no detectable reduction in tocopherol levels. However, the strain carrying the knockout for slr1736 produced no detectable levels of tocopherol.

~~Sub B57 The amino acid sequences for the *Synechocystis* knockouts are compared using ClustalW, and are provided in Table 3 below. Provided are the percent identities, percent similarity, and the percent gap. The alignment of the sequences is provided in Figure 21.~~

Table 3:

	Slr1736	slr0926	sl11899	slr0056	slr1518
slr1736 %identity	14	12	18	11	
%similar	29	30	34	26	
%gap	8	7	10	5	
slr0926 %identity		20	19	14	
%similar		39	32	28	
%gap		7	9	4	
sl11899 %identity			17	13	
%similar			29	29	
%gap			12	9	
slr0056 %identity				15	
%similar				31	
%gap				8	
slr1518 %identity					
%similar					
%gap					

~~Sub B57 Amino acid sequence comparisons are performed using various *Arabidopsis* prenyltransferase sequences and the *Synechocystis* sequences. The comparisons are presented in~~

Table 4 below. Provided are the percent identities, percent similarity, and the percent gap. The alignment of the sequences is provided in Figure 22.

Table 4:

	ATPT2	slr1736	ATPT3	slr0926	ATPT4	sll1899	ATPT12	slr0056	ATPT8	slr1518
ATPT2		29	9	9	8	8	12	9	7	9
		46	23	21	20	20	28	23	21	20
		27	13	28	23	29	11	24	25	24
slr1736			9	13	8	12	13	15	8	10
			19	28	19	28	26	33	21	26
			34	12	34	15	26	10	12	10
ATPT3				23	11	14	13	10	5	11
				36	26	26	26	21	14	22
				29	21	31	16	30	30	30
					12	20	17	20	11	14
slr0926					24	37	28	33	24	29
					33	12	25	10	11	9
						18	11	8	6	7
ATPT4						33	23	18	16	19
						28	19	32	32	33
							13	17	10	12
sll1899							24	30	23	26
							27	13	10	11
								52	8	11
ATPT1								66	19	26
2										
								18	25	23
									9	13
slr0056									23	32
									10	8

004740-81864560

T,0360

ATPT8	7
	23
	7
slr1518	

#### 4B. Preparation of the slr1737 Knockout

The *Synechocystis* sp. 6803 slr1737 knockout was constructed by the following method.

The GPS™-1 Genome Priming System (New England Biolabs) was used to insert, by a Tn7 Transposase system, a Kanamycin resistance cassette into *slr1737*. A plasmid from a *Synechocystis* genomic library clone containing 652 base pairs of the targeted orf (*Synechocystis* genome base pairs 1324051 – 1324703; the predicted orf base pairs 1323672 – 1324763, as annotated by Cyanobase) was used as target DNA. The reaction was performed according to the manufacturers protocol. The reaction mixture was then transformed into *E. coli* DH10B electrocompetant cells and plated. Colonies from this transformation were then screened for transposon insertions into the target sequence by amplifying with M13 Forward and Reverse Universal primers, yielding a product of 652 base pairs plus ~1700 base pairs, the size of the transposon kanamycin cassette, for a total fragment size of ~2300 base pairs. After this determination, it was then necessary to determine the approximate location of the insertion within the targeted orf, as 100 base pairs of orf sequence was estimated as necessary for efficient homologous recombination in *Synechocystis*. This was accomplished through amplification reactions using either of the primers to the ends of the transposon, Primer S (5' end) or N (3' end), in combination with either a M13 Forward or Reverse primer. That is, four different primer combinations were used to map each potential knockout construct: Primer S – M13 Forward, Primer S – M13 Reverse, Primer N – M13 Forward, Primer N – M13 Reverse. The construct used to transform *Synechocystis* and knockout slr1737 was determined to consist of a approximately 150 base pairs of slr1737 sequence on the 5' side of the transposon insertion and

approximately 500 base pairs on the 3' side, with the transcription of the orf and kanamycin cassette in the same direction. The nucleic acid sequence of slr1737 is provided in SEQ ID NO:38 the deduced amino acid sequence is provided in SEQ ID NO:39.

Cells of *Synechocystis* 6803 were grown to a density of  $\sim 2 \times 10^8$  cells per ml and harvested by centrifugation. The cell pellet was re-suspended in fresh BG-11 medium at a density of  $1 \times 10^9$  cells per ml and used immediately for transformation. 100 ul of these cells were mixed with 5 ul of mini prep DNA and incubated with light at 30C for 4 hours. This mixture was then plated onto nylon filters resting on BG-11 agar supplemented with TES pH8 and allowed to grow for 12-18 hours. The filters were then transferred to BG-11 agar + TES + 5ug/ml kanamycin and allowed to grow until colonies appeared within 7-10 days (Packer and Glazer, 1988). Colonies were then picked into BG-11 liquid media containing 5 ug/ml kanamycin and allowed to grow for 5 days. These cells were then transferred to Bg-11 media containing 10ug/ml kanamycin and allowed to grow for 5 days and then transferred to Bg-11 + kanamycin at 25ug/ml and allowed to grow for 5 days. Cells were then harvested for PCR analysis to determine the presence of a disrupted ORF and also for HPLC analysis to determine if the disruption had any effect on tocopherol levels.

PCR analysis of the *Synechocystis* isolates, using primers to the ends of the *slr1737* orf , showed complete segregation of the mutant genome, meaning no copies of the wild type genome could be detected in these strains. This suggests that function of the native gene is not essential for cell function. HPLC analysis of the strain carrying the knockout for *slr1737* produced no detectable levels of tocopherol.

#### 4C. Phytyl Prenyltransferase Enzyme Assays

[ $^3\text{H}$ ] Homogentisic acid in 0.1%  $\text{H}_3\text{PO}_4$  (specific radioactivity 40 Ci/mmol). Phytyl pyrophosphate was synthesized as described by Joo, *et al.* (1973) *Can J. Biochem.* 51:1527. 2-methyl-6-phytylquinol and 2,3-dimethyl-5-phytylquinol were synthesized as described by Soll, *et al.* (1980) *Phytochemistry* 19:215. Homogentisic acid,  $\alpha$ ,  $\beta$ ,  $\delta$ , and  $\gamma$ -tocopherol, and tocol, were purchased commercially.

The wild-type strain of *Synechocystis* sp. PCC 6803 was grown in BG11 medium with bubbling air at 30°C under 50  $\mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$  fluorescent light, and 70% relative humidity. The growth

medium of slr1736 knock-out (potential PPT) strain of this organism was supplemented with 25  $\mu\text{g mL}^{-1}$  kanamycin. Cells were collected from 0.25 to 1 liter culture by centrifugation at 5000g for 10 min and stored at  $-80^{\circ}\text{C}$ .

Total membranes were isolated according to Zak's procedures with some modifications (Zak, *et al.* (1999) *Eur J. Biochem* 261:311). Cells were broken on a French press. Before the French press treatment, the cells were incubated for 1 hour with lysozyme (0.5%, w/v) at  $30^{\circ}\text{C}$  in a medium containing 7 mM EDTA, 5 mM NaCl and 10 mM Hepes-NaOH, pH 7.4. The spheroplasts were collected by centrifugation at 5000g for 10 min and resuspended at 0.1 - 0.5 mg chlorophyll- $\text{mL}^{-1}$  in 20 mM potassium phosphate buffer, pH 7.8. Proper amount of protease inhibitor cocktail and DNAase I from Boehringer Mannheim were added to the solution. French press treatments were performed two to three times at 100 MPa. After breakage, the cell suspension was centrifuged for 10 min at 5000g to pellet unbroken cells, and this was followed by centrifugation at 100 000 g for 1 hour to collect total membranes. The final pellet was resuspended in a buffer containing 50 mM Tris-HCL and 4 mM  $\text{MgCl}_2$ .

Chloroplast pellets were isolated from 250 g of spinach leaves obtained from local markets. Devined leaf sections were cut into grinding buffer (2 1/250 g leaves) containing 2 mM EDTA, 1 mM  $\text{MgCl}_2$ , 1 mM  $\text{MnCl}_2$ , 0.33 M sorbitol, 0.1% ascorbic acid, and 50 mM Hepes at pH 7.5. The leaves were homogenized for 3 sec three times in a 1-L blender, and filtered through 4 layers of miracloth. The supernatant was then centrifuged at 5000g for 6 min. The chloroplast pellets were resuspended in small amount of grinding buffer (Douce, *et al* Methods in Chloroplast Molecular Biology, 239 (1982)

Chloroplasts in pellets can be broken in three ways. Chloroplast pellets were first aliquoted in 1 mg of chlorophyll per tube, centrifuged at 6000 rpm for 2 min in microcentrifuge, and grinding buffer was removed. Two hundred microliters of Triton X-100 buffer (0.1% Triton X-100, 50 mM Tris-HCl pH 7.6 and 4 mM  $\text{MgCl}_2$ ) or swelling buffer (10 mM Tris pH 7.6 and 4 mM  $\text{MgCl}_2$ ) was added to each tube and incubated for 1/2 hour at  $4^{\circ}\text{C}$ . Then the broken chloroplast pellets were used for the assay immediately. In addition, broken chloroplasts can also be obtained by freezing in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  for 1/2 hour, then used for the assay.

In some cases chloroplast pellets were further purified with 40%/ 80% percoll gradient to obtain intact chloroplasts. The intact chloroplasts were broken with swelling buffer, then either

used for assay or further purified for envelope membranes with 20.5%/ 31.8% sucrose density gradient (Sol, *et al* (1980) *supra*). The membrane fractions were centrifuged at 100 000g for 40 min and resuspended in 50 mM Tris-HCl pH 7.6, 4 mM MgCl<sub>2</sub>.

Various amounts of [<sup>3</sup>H]HGA, 40 to 60 μM unlabelled HGA with specific activity in the range of 0.16 to 4 Ci/mmol were mixed with a proper amount of 1M Tris-NaOH pH 10 to adjust pH to 7.6. HGA was reduced for 4 min with a trace amount of solid NaBH<sub>4</sub>. In addition to HGA, standard incubation mixture (final vol 1 mL) contained 50 mM Tris-HCl, pH 7.6, 3-5 mM MgCl<sub>2</sub>, and 100 μM phytyl pyrophosphate. The reaction was initiated by addition of *Synechocystis* total membranes, spinach chloroplast pellets, spinach broken chloroplasts, or spinach envelope membranes. The enzyme reaction was carried out for 2 hour at 23°C or 30°C in the dark or light. The reaction is stopped by freezing with liquid nitrogen, and stored at -80°C or directly by extraction.

A constant amount of tocol was added to each assay mixture and reaction products were extracted with a 2 mL mixture of chloroform/methanol (1:2, v/v) to give a monophasic solution. NaCl solution (2 mL; 0.9%) was added with vigorous shaking. This extraction procedure was repeated three times. The organic layer containing the prenylquinones was filtered through a 20 μm filter, evaporated under N<sub>2</sub>, and then resuspended in 100 μL of ethanol.

The samples were mainly analyzed by Normal-Phase HPLC method (Isocratic 90% Hexane and 10% Methyl-t-butyl ether), and use a Zorbax silica column, 4.6 x 250 mm. The samples were also analyzed by Reversed-Phase HPLC method (Isocratic 0.1% H<sub>3</sub>PO<sub>4</sub> in MeOH), and use a Vydac 201HS54 C18 column; 4.6 x 250 mm coupled with an All-tech C18 guard column. The amount of products were calculated based on the substrate specific radioactivity, and adjusted according to the % recovery based on the amount of internal standard.

The amount of chlorophyll was determined as described in Arnon (1949) *Plant Physiol.* 24:1. Amount of protein was determined by the Bradford method using gamma globulin as a standard (Bradford, (1976) *Anal. Biochem.* 72:248)

Results of the assay demonstrate that 2-Methyl-6-Phytylplastoquinone is produced in the *Synechocystis* slr1736 knockout preparations. The results of the phytyl prenyltransferase enzyme activity assay for the slr1736 knock out are presented in Figure 23.

4D. Complementation of the slr1736 knockout with ATPT2

In order to determine whether ATPT2 could complement the knockout of slr1736 in *Synechocystis 6803* a plasmid was constructed to express the ATPT2 sequence from the TAC promoter. A vector, plasmid psl1211, was obtained from the lab of Dr. Himadri Pakrasi of Washington University, and is based on the plasmid RSF1010 which is a broad host range plasmid (Ng W.-O., Zentella R., Wang, Y., Taylor J-S. A., Pakrasi, H.B. 2000. *phrA*, the major photoreactivating factor in the cyanobacterium *Synechocystis* sp. strain PCC 6803 codes for a cyclobutane pyrimidine dimer specific DNA photolyase. *Arch. Microbiol.* (in press)). The ATPT2 gene was isolated from the vector pCGN10817 by PCR using the following primers. ATPT2nco.pr 5'-CCATGGATTTCGAGTAAAGTTGTCGC (SEQ ID NO:89); ATPT2ri.pr- 5'-GAATTCACCTTCAAAAAAGGTAACAG (SEQ ID NO:90). These primers will remove approximately 112 BP from the 5' end of the ATPT2 sequence, which is thought to be the chloroplast transit peptide. These primers will also add an NcoI site at the 5' end and an EcoRI site at the 3' end which can be used for sub-cloning into subsequent vectors. The PCR product from using these primers and pCGN10817 was ligated into pGEM T easy and the resulting vector pMON21689 was confirmed by sequencing using the m13forward and m13reverse primers. The NcoI/EcoRI fragment from pMON21689 was then ligated with the EagI/EcoRI and EagI/NcoI fragments from psl1211 resulting in pMON21690. The plasmid pMON21690 was introduced into the slr1736 *Synechocystis 6803* KO strain via conjugation. Cells of sl906 (a helper strain) and DH10B cells containing pMON21690 were grown to log phase (O.D. 600=0.4) and 1 ml was harvested by centrifugation. The cell pellets were washed twice with a sterile BG-11 solution and resuspended in 200 ul of BG-11. The following was mixed in a sterile eppendorf tube: 50 ul SL906, 50 ul DH10B cells containing pMON21690, and 100 ul of a fresh culture of the slr1736 *Synechocystis 6803* KO strain (O.D. 730 = 0.2-0.4). The cell mixture was immediately transferred to a nitrocellulose filter resting on BG-11 and incubated for 24 hours at 30C and 2500 LUX(50 ue) of light. The filter was then transferred to BG-11 supplemented with 10ug/ml Gentamycin and incubated as above for ~5 days. When colonies appeared, they were picked and grown up in liquid BG-11 + Gentamycin 10 ug/ml. (Elhai, J. and Wolk, P. 1988. Conjugal transfer of DNA to Cyanobacteria. *Methods in Enzymology* 167, 747-54) The liquid cultures were then assayed for tocopherols by harvesting 1ml of culture by centrifugation,



extracting with ethanol/pyrogallol, and HPLC separation. The slr1736 *Synechocystis* 6803 KO strain, did not contain any detectable tocopherols, while the slr1736 *Synechocystis* 6803 KO strain transformed with pmon21690 contained detectable alpha tocopherol. A *Synechocystis* 6803 strain transformed with psl1211(vector control) produced alpha tocopherol as well.

5

# **Example 5: Transgenic Plant Analysis**

Arabidopsis plants transformed with constructs for the sense or antisense expression of the ATPT proteins were analyzed by High Pressure Liquid Chromatography (HPLC) for altered levels of total tocopherols, as well as altered levels of specific tocopherols (alpha, beta, gamma, and delta tocopherol).

Extracts of leaves and seeds were prepared for HPLC as follows. For seed extracts, 10 mg of seed was added to 1 g of microbeads (Biospec) in a sterile microfuge tube to which 500 ul 1% pyrogallol (Sigma Chem)/ethanol was added. The mixture was shaken for 3 minutes in a mini Beadbeater (Biospec) on "fast" speed. The extract was filtered through a 0.2 um filter into an autosampler tube. The filtered extracts were then used in HPLC analysis described below.

Leaf extracts were prepared by mixing 30-50 mg of leaf tissue with 1 g microbeads and freezing in liquid nitrogen until extraction. For extraction, 500 ul 1% pyrogallol in ethanol was added to the leaf/bead mixture and shaken for 1 minute on a Beadbeater (Biospec) on "fast" speed. The resulting mixture was centrifuged for 4 minutes at 14,000 rpm and filtered as described above prior to HPLC analysis.

HPLC was performed on a Zorbax silica HPLC column (4.6 mm X 250 mm) with a fluorescent detection, an excitation at 290 nm, an emission at 336 nm, and bandpass and slits. Solvent A was hexane and solvent B was methyl-t-butyl ether. The injection volume was 20 ul, the flow rate was 1.5 ml/min, the run time was 12 min (40°C) using the gradient (Table 5):

**Table 5:**

<u>Time</u>	<u>Solvent A</u>	<u>Solvent B</u>
0 min.	90%	10%
10 min.	90%	10%
11 min.	25%	75%
12 min.	90%	10%

Tocopherol standards in 1% pyrogallol/ ethanol were also run for comparison (alpha tocopherol, gamma tocopherol, beta tocopherol, delta tocopherol, and tocopherol (tocol) (all from Matreya).

Standard curves for alpha, beta, delta, and gamma tocopherol were calculated using Chemstation software. The absolute amount of component x is: Absolute amount of x =  $\text{Response}_x \times \text{RF}_x \times \text{dilution factor}$  where  $\text{Response}_x$  is the area of peak x,  $\text{RF}_x$  is the response factor for component x ( $\text{Amount}_x / \text{Response}_x$ ) and the dilution factor is 500 ul. The ng/mg tissue is found by: total ng component/mg plant tissue.

Results of the HPLC analysis of seed extracts of transgenic *Arabidopsis* lines containing pMON10822 for the expression of ATAT2 from the napin promoter are provided in Figure 24.

HPLC analysis results of *Arabidopsis* seed tissue expressing the ATAT2 sequence from the napin promoter (pMON10822) demonstrates an increased level of tocopherols in the seed. Total tocopherol levels are increased as much as 50 to 60% over the total tocopherol levels of non-transformed (wild-type) *Arabidopsis* plants (Figure 24).

Furthermore, increases of particular tocopherols are also increased in transgenic *Arabidopsis* plants expressing the ATAT2 nucleic acid sequence from the napin promoter. Levels of delta tocopherol in these lines are increased greater than 3 fold over the delta tocopherol levels obtained from the seeds of wild type *Arabidopsis* lines. Levels of gamma tocopherol in transgenic *Arabidopsis* lines expressing the ATAT2 nucleic acid sequence are increased as much as about 60% over the levels obtained in the seeds of non-transgenic control lines. Furthermore, levels of alpha tocopherol are increased as much as 3 fold over those obtained from non-transgenic control lines.

Results of the HPLC analysis of seed extracts of transgenic *Arabidopsis* lines containing pMON10803 for the expression of ATAT2 from the enhanced 35S promoter are provided in Figure 25.

All publications and patent applications mentioned in this specification are indicative of the level of skill of those skilled in the art to which this invention pertains. All publications and

patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

- Although the foregoing invention has been described in some detail by way of illustration  
5 and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claim.

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